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The ActA Polypeptides of *Listeria ivanovii* and *Listeria monocytogenes* Harbor Related Binding Sites for Host Microfilament Proteins

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The surface-bound ActA polypeptide of the intracellular bacterial pathogen *Listeria monocytogenes* acts as a nucleator protein, generating the actin cytoskeleton around intracellularly motile bacteria. In this work, we examined the functional similarity of ActA from *Listeria ivanovii* (iActA) ATCC 19119 to its *L. monocytogenes* counterpart. The amino acid sequence of iActA predicts a molecular mass of 123 kDa and harbors eight proline-rich repeats. For functional analysis, various iActA derivatives and hybrid constructs of *L. ivanovii* and *L. monocytogenes* ActA polypeptides were transiently expressed in epithelial cells and examined for recruitment of host microfilament proteins by a mitochondrial targeting assay. As has been demonstrated with ActA, iActA also spontaneously inserted into the surface of mitochondria and induced recruitment of actin, α-actinin, and the vasodilator-stimulated phosphoprotein (VASP) to these subcellular organelles. By comparison of amino-terminally truncated iActA derivatives for their ability to recruit cytoskeletal proteins, a region essential for actin filament accumulation was identified between amino acid residues 290 and 325. Such derivatives, however, retained their ability to bind VASP. Replacement of the proline-rich repeats in ActA with those of iActA also resulted in VASP recruitment. Hence, despite the limited overall sequence homology between ActA and iActA, the two molecules consist of at least two similar domains: a highly positively charged N-terminal domain that is directly involved in actin filament recruitment and a proline-rich repeat region required for VASP binding.

Within the bacterial genus *Listeria*, *Listeria monocytogenes* and *L. ivanovii* are the only pathogenic species. Whereas *L. monocytogenes* causes severe infections in humans and animals, *L. ivanovii* infections are largely restricted to animals, in particular sheep (25). In tissue culture invasion assays, both species are capable of penetrating a wide range of host cells and multiplying within their cytoplasm. Following escape from the phagolysosomal compartment, these bacteria begin to accumulate actin filaments on their surface. These actin "clouds" subsequently form a polarized actin tail at one bacterial pole that enables these pathogens to move intracellularly and to spread from one cell to another (8, 17, 30). In *L. monocytogenes*, the products encoded by a virulence gene cluster have been implicated in the process of escape from the phagolysosome, intracellular movement, and cell-to-cell spread (22). Recently, an analogous virulence gene cluster has been identified in *L. ivanovii* (6, 7).

The *actA* gene product of *L. monocytogenes* has been identified as an essential factor required for actin accumulation, since mutants lacking ActA do not recruit actin filaments and as a result grow as microcolonies in infected host cells (3, 9). Expression in eukaryotic cells demonstrated that ActA, when targeted to either mitochondria or the inner surface of the plasma membrane, induces local accumulation of actin filaments (5, 20, 21). In addition, coating of *Streptococcus pneumonia* cells with purified ActA and expression of ActA in nonpathogenic *Listeria innocua* confer actin-based motility to these otherwise nonmotile bacteria upon incubation in *Xenopus* egg extracts (10, 27). Hence, ActA alone is sufficient to initiate the reorganization of the actin cytoskeleton. Furthermore, ActA contains at least two essential sites that are required for efficient microfilament assembly: an amino-terminal region for actin filament nucleation and a central proline-rich repeat region that binds directly to the vasodilator-stimulated phosphoprotein (VASP) (20, 21). Recently, the *actA* gene of *L. ivanovii* (*iactA*) has been described (6, 12). These data have revealed that iActA is considerably larger than ActA and that there is limited overall homology in their primary sequences. Since the two polypeptides are required for intracellular motility of the respective bacteria, we sought to identify the regions in the iActA polypeptide that are required for VASP binding and actin filament recruitment.

MATERIALS AND METHODS

Bacterial strains, media, and reagents. The hemolytic *L. monocytogenes* strain EGD (serotype 1/2 a) and its isogenic ActA deletion mutant (Δ*actA2*) have been described previously (1, 2), as well as *L. ivanovii* ATCC 19119 (8). The *L. ivanovii* wild-type strains SLCC 4770 and SLCC 5378 were kindly provided by H. Hof and T. Nichterlein, University of Heidelberg, Heidelberg, Germany.

All strains were routinely grown in brain heart infusion broth (Difco) at 37°C. The *L. monocytogenes* Δ*actA2* mutant harboring plasmid pERL3 50-1 or pBPLIVAN was grown in the presence of 5 μg of erythromycin per ml.

Restriction endonucleases, *Taq* polymerase sequencing reagents, and ligase were purchased from Biolabs (Schwalbach, Germany) and Perkin-Elmer (Ueberlingen, Germany) and used according to the manufacturers' instructions. All other chemical reagents were purchased from Sigma (Deisenhofen, Germany) unless indicated otherwise.

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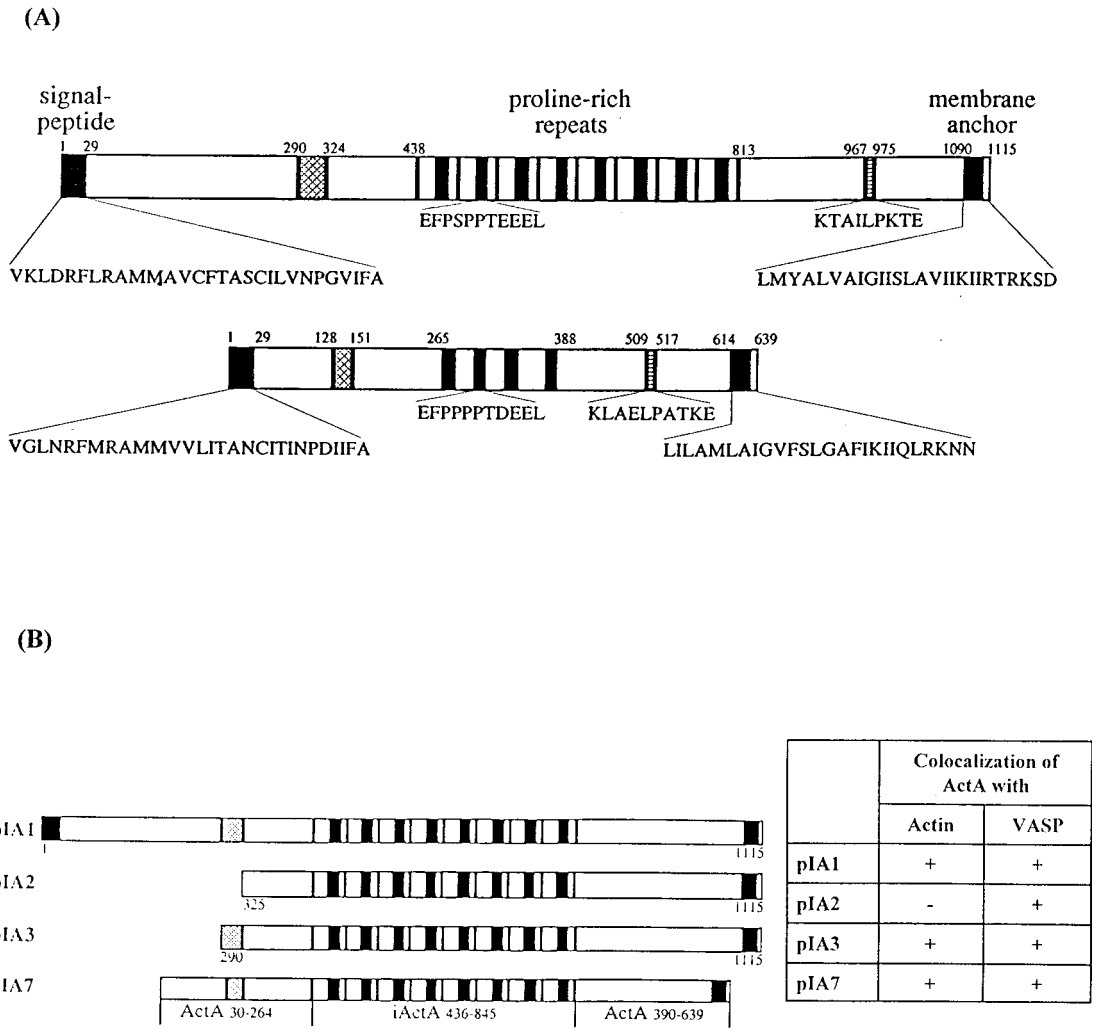

FIG. 1. (A) Schematic representation of the iActA polypeptide of *L. ivanovii* ATCC 19119 (above) and the ActA polypeptide of *L. monocytogenes* EGD (below). The positions and sequences of various functional domains, including the putative domains required for actin filament recruitment, are indicated. (B) Schematic representation of various iActA derivatives used in this study. All derivatives were targeted to mitochondria. (C) Alignment of the putative actin filament-recruiting regions of ActA and iActA. (D) Alignment of the amino acids in the N-terminal end of *L. ivanovii* ATCC 19119 (for details, see Materials and Methods). (E) Alignment of the conserved proline-rich repeats (R) of the ActA polypeptides of *L. monocytogenes* EGD and the three *L. ivanovii* strains. Repeats are numbered according to their positions within the individual repeat regions.

Cloning and analysis of *actA* derivatives from *L. ivanovii* and *L. monocytogenes*. The following oligonucleotides were used as primers for the amplification of the *actA* gene from genomic DNAs derived from different *L. ivanovii* strains or *L. monocytogenes* EGD (restriction sites introduced at the 5′ and 3′ ends are indicated in boldface): A, 5′-GGCGGAAGAA**GTCGAC**AAAAAAGTAGGAG AA-3′; B, 5′-ACCAAG**GGATCC**TTTGTTGATTCTCATAGC-3′; C, 5′-AAT **GTCGAC**GAGGAGTAAAAAAATGAAACTAGATAG-3′; D, 5′-CCCAA**AA GCTT**TATGAAAGAAATAT-3′; E, 5′-GATATG**GTCGAC**TCTGATATGGA AG-3′; F, 5′-GACATA**AAGCTT**GCAATGACACAGATAGCG-3′; G, 5′-TGG CGGG**GGATCC**GAAGCATTTAC-3′; H, 5′-CAG**CTGATCA**ATCATTGGAA TTGGG-3′; I, 5′-CTGTAT**GGATCC**CATAGAAATATGAAC-3′; K, 5′-GAAG AAG**AGATCT**ACGGGAGAGGC-3′; and L, 5′-TTTGAATTTC**GGATCC**T TCACCTCATT-3′.

The complete *actA* gene of *L. ivanovii* ATCC 19119, including its promoter, was inserted into the modified pERL3 50-1 vector (2, 14). The vector was modified by insertion of a new polylinker, generating unique *Sal*I, *Xho*I, and *Bgl*II restriction sites. The resulting vector was cut with *Bgl*II and *Sal*I to insert the *actA* gene of *L. ivanovii* as a *Sal*I-*Bam*HI fragment to generate the plasmid pBPLIVAN. The *iactA* gene was amplified from genomic DNA of *L. ivanovii* ATCC 19119 by PCR using primers A and B according to the published *iactA* sequence (12). For detailed sequencing of the complete *iactA* gene, this plasmid was digested with *Eco*RI and the resulting *Eco*RI fragments were subcloned into pUC18 (cut with *Eco*RI). Sequencing primers were universal 17-mer sequencing primer 1211 (Biolabs) and 24-mer reverse sequencing primer 1233 (Biolabs).

For eukaryotic expression, the *iactA* gene of *L. ivanovii* ATCC 19119 was inserted into the eukaryotic expression vector pMPSVHE (20). Sequences 5′ to the *iactA* gene were modified to allow expression in eukaryotic cells (11) and cloned into the pMPSVHE vector by using *Sal*I-*Bam*HI restriction sites introduced into regions flanking the gene by specific primers. The *iactA* gene was amplified from genomic DNA of *L. ivanovii* ATCC 19119 by PCR using primers B and C. The resulting plasmid was termed pIA1.

For generation of the N-terminal truncated version of iActA starting with amino acid (aa) 325, primers B and E were used to amplify the *iactA* gene from genomic DNA of *L. ivanovii* ATCC 19119. Restriction sites at the 5′ end (*Hin*dIII) and 3′ end (*Bam*HI) were used to generate cohesive ends to ligate this fragment into the pMPSVHE vector cut with *Hin*dIII and *Bam*HI, respectively. The resulting plasmid, pIA2, contained the truncated iActA protein starting at aa 325. Plasmid pIA3 started at aa 290, and primers B and D were used for amplification of this *iactA* derivative. The restriction sites at the 5′ end (*Sal*I) and 3′ end (*Bam*HI) were digested, and the fragments were ligated into the pMPSVHE vector cut with *Sal*I and *Bam*HI, respectively.

We used the following cloning strategy to generate pIA7. The N-terminal region up to the proline-rich repeat of *L. monocytogenes* ActA (see Fig. 1A) was amplified from genomic DNA of *L. monocytogenes* EGD with primers F and G. The fragment was digested with *Hin*dIII and *Bam*HI and inserted into a *Hin*dIII-*Bam*HI-digested pMPSVHE vector. This construct was digested with *Bam*HI and ligated to a PCR fragment which contained the proline-rich repeat region of *L. ivanovii iactA* amplified from genomic DNA of *L. ivanovii* ATCC 19119 with

(C)

ActA (*L. monocytogenes* EGD) $_{129}$QVERRHPGLPSDSAAE**IKKRRKAIASSDSELESLTYPDKP**$_{168}$

iActA(*L. ivanovii* ATCC19119) $_{292}$EILSDEQNRVPMNSGK**IKNRRKAIEGSDME**DSDMEDA**DTE**$_{331}$

(D)

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39 EN-SNLESDEQGEGEQAEGKVEEGR-----NSPGHGAISEACARDIQELGKIGEAKSANVPDSMTTPDSGLSKEPGQNIGET--------- 115
   :| | :||| :|| | ||||| |: | |||| || ||| | | |:||| || | |:| ||
193 DNDSRIDSDEWDDGEEAKEKVEEGKAEEEKNNLGQEEISEARERDLQELEKMGKVKNANVTALLAMLDSRAGKVARQDIKETLNDEVPAVF 283
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(E)

ATCC 19119	(R1/R3/R4/R5/R6/R7/R8)	**EFPSPPTEEEL**
SLCC 4770	(R1/R6)	**EFPSPPTEEEL**
SLCC 5378	(R1/R2/R4/R5)	**EFPSPPTEEEL**
EGD	(R1)	**DFPPPPTDEEL**
EGD	(R2)	**EFPPPPTDEEL**
EGD	(R3)	**EFPPPPTEDEL**
EGD	(R4)	**DFPPIPTEEEL**

FIG. 1—*Continued.*

primers H and I (cut with *Bcl*I and *Bam*HI). After the orientation of the fragment was controlled, the resulting construct was again digested with *Bam*HI and ligated to the C-terminal part of *L. monocytogenes* ActA (see Fig. 1A), which had been amplified from genomic DNA of *L. monocytogenes* with primers K and L and digested with *Bgl*II and *Bam*HI.

All inserts of the constructs were verified by sequencing with Taa Dye Deoxy Terminator cycle sequencing (Applied Biosystems) and analyzed on an Applied Biosystems 373A automated DNA sequencer.

Amplification of the *iactA* proline-rich repeat regions of different *L. ivanovii* strains. Primers H and I were used to amplify the *iactA* proline-rich repeat regions of the different *L. ivanovii* strains. 5′-amplification primer H has multiple binding sites downstream of the nucleotides coding for the proline-rich regions. 3′-amplification primer I binds to a unique region upstream of the nucleotides which code for the proline-rich region.

For further characterization of the *iactA* proline-rich regions of *L. ivanovii* SLCC 4770 and SLCC 5378, these regions were amplified with the primers 5′-CTCATCATCTGTATGGAATCCATA-3′ and 5′-AGTGAACCTAGTTCT TTTAATCTC-3′. The amplified products were sequenced with the same primers to analyze the repeat motifs of the different *L. ivanovii* strains.

For determination of the length of the *iactA* genes of *L. ivanovii* SLCC 4770 and SLCC 5378 in comparison with that of *L. ivanovii* ATCC 19119, the complete coding sequences of these strains were amplified with primers B and C. The primers were complementary to the 5′ and 3′ noncoding regions of the *iactA* sequence (12). The resulting PCR products were analyzed on a 2% agarose gel to obtain the actual sizes.

Preparation of SDS cell wall extracts of different *Listeria* strains. Exponentially growing cultures were harvested by centrifugation, and bacterial pellets were washed twice with phosphate-buffered saline (PBS) and incubated with 1% (wt/vol) sodium dodecyl sulfate (SDS) in PBS for 1 h at 37°C (9, 18).

SDS-PAGE, immunoblotting, ligand overlay, and protein sequence analysis. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 7.5% polyacrylamide gels which were either stained with Coomassie brilliant blue R-250 or transblotted onto Immobilon P membranes (Millipore) with a semidry device. Filters were incubated with affinity-purified polyclonal ActA antibodies (18) and secondary peroxidase-conjugated goat anti-rabbit antibodies (Dianova, Hamburg, Germany). Immunoblots were developed with an enhancing chemiluminescence (ECL) system (Amersham, Braunschweig, Germany).

For the ligand overlay blot assay, samples were separated by SDS-PAGE and blotted onto Immobilon P membranes (Millipore). The membranes were incubated with ^{32}P-labelled VASP prepared and processed as described previously (1). For the N-terminal protein microsequencing, the proteins were transblotted onto MiniProBlot membranes (Applied Biosystems) and were analyzed with an Applied Biosystems gas phase sequenator (model A470) equipped with an online phenylthiohydantoin amino acid analyzer.

Expression studies and immunofluorescence microscopy. For expression of iActA of *L. ivanovii* in *L. monocytogenes* Δ*actA2*, the plasmid pBPLIVAN was transformed into electrocompetent *L. monocytogenes* Δ*actA2* cells (19).

PtK_2 cells (ATCC CCL 56) were grown and transfected as described previously (20, 21). For transfection studies, PtK_2 cells were grown as monolayers on coverslips. Mitochondrial staining was performed with a rhodamine-labelled Mitotracker CMTMRos (Molecular Probes) used at a concentration of 0.2 μg/ml in complete medium and incubated for 1 h at 37°C before the cells were fixed with 3.7% formaldehyde (vol/vol) in PBS. For the localization of ActA, α-actinin, VASP, and actin, the cells were fixed with methanol or 3.7% formaldehyde in PBS, permeabilized, and processed for double-immunofluorescence microscopy as described previously (21). The polyclonal antibodies against ActA and against VASP as well as the monoclonal antibodies against ActA (N20 and S188) have been described previously (18, 24). The α-actinin antibody (A5044) was from Sigma, the monoclonal actin antibody (C4) was from Boehringer, and the rhodamine- and fluorescein-labelled secondary antibodies were purchased from Dianova. Samples were examined with a Zeiss Axiophot microscope equipped with epifluorescence. Photographs were taken with Kodak T-MAX 400 film.

Computer-supported sequence analysis. Alignments were performed with the program Geneworks, version 2.45, from IntelliGenetics. The protein alignment of this program uses a PAM-250 scoring matrix. The cost to open a gap was 5, and the cost to lengthen a gap was 25 (15, 28).

RESULTS

Analysis of the *iactA* gene of *L. ivanovii*. In order to perform functional studies with the ActA polypeptide of *L. ivanovii* (iActA), we amplified the *iactA* gene by PCR using oligonu-

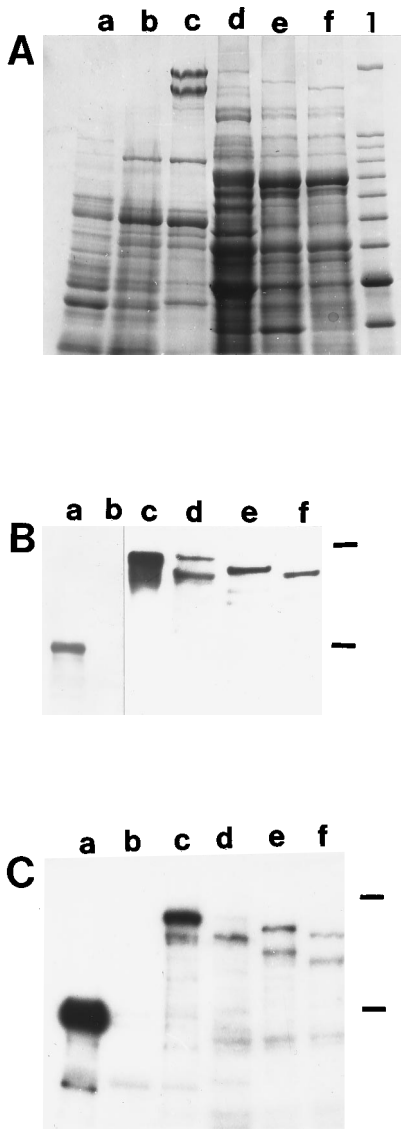

FIG. 2. Detection of ActA polypeptides in SDS cell wall extracts of different *Listeria* strains. (A) Extracts (lanes a to f) were separated by SDS-PAGE (7.5% polyacrylamide) and stained with Coomassie brilliant blue. Lane a, *L. monocytogenes* EGD; lane b, Δ*actA2* deletion mutant of *L. monocytogenes* EGD complemented with pERL3 50-1; lane c, *L. monocytogenes* Δ*actA2* complemented with pBPLIVAN; lane d, *L. ivanovii* ATCC 19119; lane e, *L. ivanovii* SLCC 4770; and lane f, *L. ivanovii* SLCC 5378. Increased amounts of protein have been loaded in lanes d to f in order to detect the different iActA polypeptides. Lane 1, molecular mass marker (top to bottom: 200, 120, 110, 100, 90, 80, 70, 60, 50, and 40 kDa). (B) Corresponding immunoblot reacted with the affinity-purified polyclonal ActA antibodies and developed with a sensitive chemiluminescent substrate. Because of the restricted cross-reactivity of the ActA antibodies with iActA, lane a was exposed less. (C) Corresponding ligand overlay performed with ^{32}P-labelled VASP. Note that iActA binds VASP more weakly than ActA. Positions of molecular mass markers of 200 and 90 kDa are indicated to the right of panels B and C (bars).

cleotides flanking the coding sequence (see Materials and Methods). Genomic DNA derived from *L. ivanovii* ATCC 19119 was used as a template. Sequencing of the *iactA* gene of *L. ivanovii* ATCC 19119 revealed a polypeptide of 1,115 aa harboring eight identical proline-rich motifs. The polypeptide had a predicted size of approximately 123 kDa, but it showed an aberrant mobility on SDS gels, running at approximately 190 kDa (see Fig. 2A). One additional proline-rich repeat was identified in the iActA sequence of this strain in comparison with the published sequence of strain CLIP 257 (6). Otherwise, the sequences of the two strains are identical. We have preferred to assign valine as the N-terminal amino acid for iActA as this residue is preceded by a consensus ribosome-binding site.

The resulting first 29 aa of the deduced sequence of iActA showed significant homology to the ActA signal sequence of *L. monocytogenes* (3, 9) (Fig. 1A). In mature iActA, the N-terminal region preceding the proline-rich repeats is significantly longer than that of *L. monocytogenes* ActA (ActA). Computer-supported sequence analysis of this region of iActA (aa 30 to 293) suggested that this portion of the polypeptide arose as a result of a duplication, as shown in the alignment in Fig. 1C. An N-terminally located, highly positively charged consensus sequence within a region recently identified as being essential for the accumulation of actin filaments by ActA (21) is also present in iActA (Fig. 1B). The distances between this sequence and the proline-rich repeat regions in ActA and iActA are similar (120 versus 155 aa).

The C-terminal end of iActA has features of a typical membrane anchor, including hydrophobic residues followed by positively charged ones. At amino acid positions 967 to 975, iActA also contains a sequence motif typical for surface proteins of gram-positive bacteria (KTAILPKTEP; Fig. 1A) (see also reference 4). Apart from these features, the sequences of the C termini of the two ActA polypeptides are very similar in length but have limited homology. The positions of the various functional motifs of both ActA polypeptides are schematically shown in Fig. 1A.

Heterogeneity of the iActA polypeptides in various *L. ivanovii* strains. Immunoblot analysis of SDS cell wall extracts from three *L. ivanovii* strains (ATCC 19119, SLCC 4770, and SLCC 5378) showed that these strains expressed ActA polypeptides with different molecular weights (Fig. 2A and B). Comparative analysis of the central ActA repeat regions of these strains with PCR primers designed to amplify consecutive repeats (see Materials and Methods) showed that the different molecular weights of the ActA polypeptides could be directly correlated with the sizes of the amplified fragments. Thus, six repeats were predicted for SLCC 4770, five were predicted for SLCC 5378, and eight were predicted for ATCC 19119 (Fig. 3, lanes

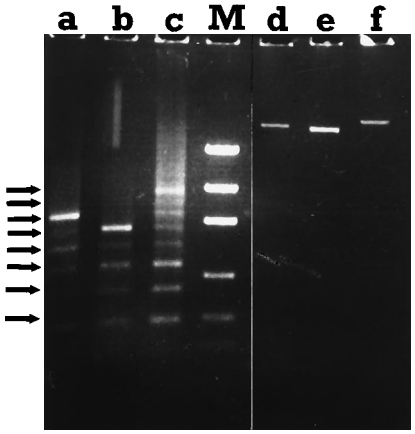

FIG. 3. Analysis of the *iactA* genes of different *L. ivanovii* strains by PCR. Lanes a to c, amplification of the proline-rich repeat regions; lanes d to f, amplification of the complete *iactA* coding regions. Lanes a and d, SLCC 4770; lanes b and e, SLCC 5378; lanes c and f, ATCC 19119. Lane M, molecular size markers of 2.0, 1.2, 0.8, 0.4, and 0.2 kb (from top to bottom). Positions of single repeats are indicated on the left (arrows).

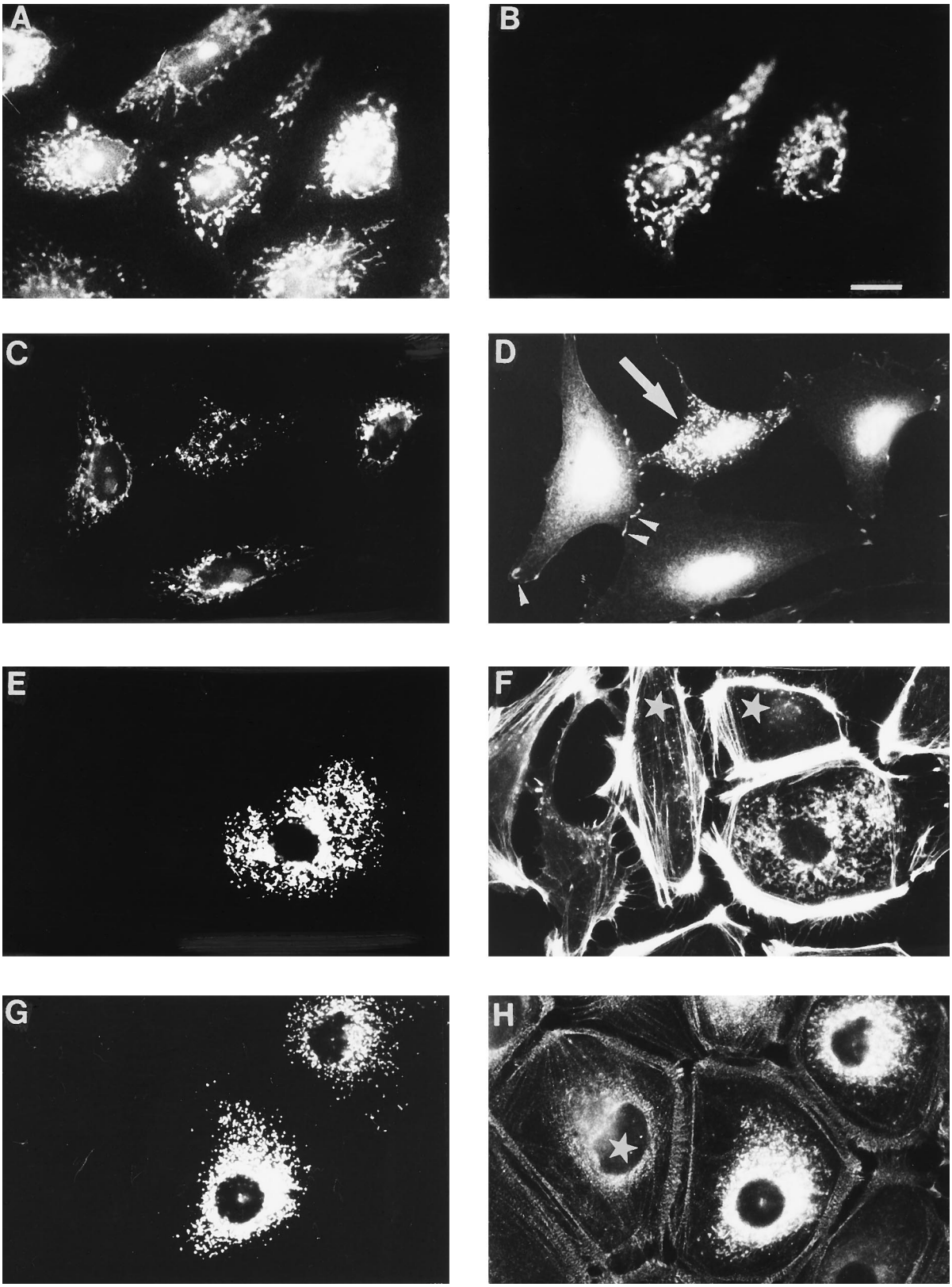

FIG. 4. Analysis of mitochondrially targeted iActA in transiently transfected PtK_2 cells. Twenty-four hours after the start of transfection, cells transfected with plasmid pIA1 were fixed with formaldehyde (A through D) or methanol (E through H) and processed for double-immunofluorescence microscopy. Images were obtained with the Mitotracker CMTMRos (A and C) (see Materials and Methods), polyclonal ActA antibodies (B, E, and G), monoclonal actin antibodies (F), monoclonal α-actinin antibodies (H), or polyclonal VASP antibodies (D). VASP localization in focal contacts of nontransfected cells (arrowheads) and the transfected cell (arrow) are indicated in panel D. The diffuse VASP staining in panel D is due to the formaldehyde fixation procedure. Nontransfected cells in panels F and H (stars) are indicated. Bar, 10 μm.

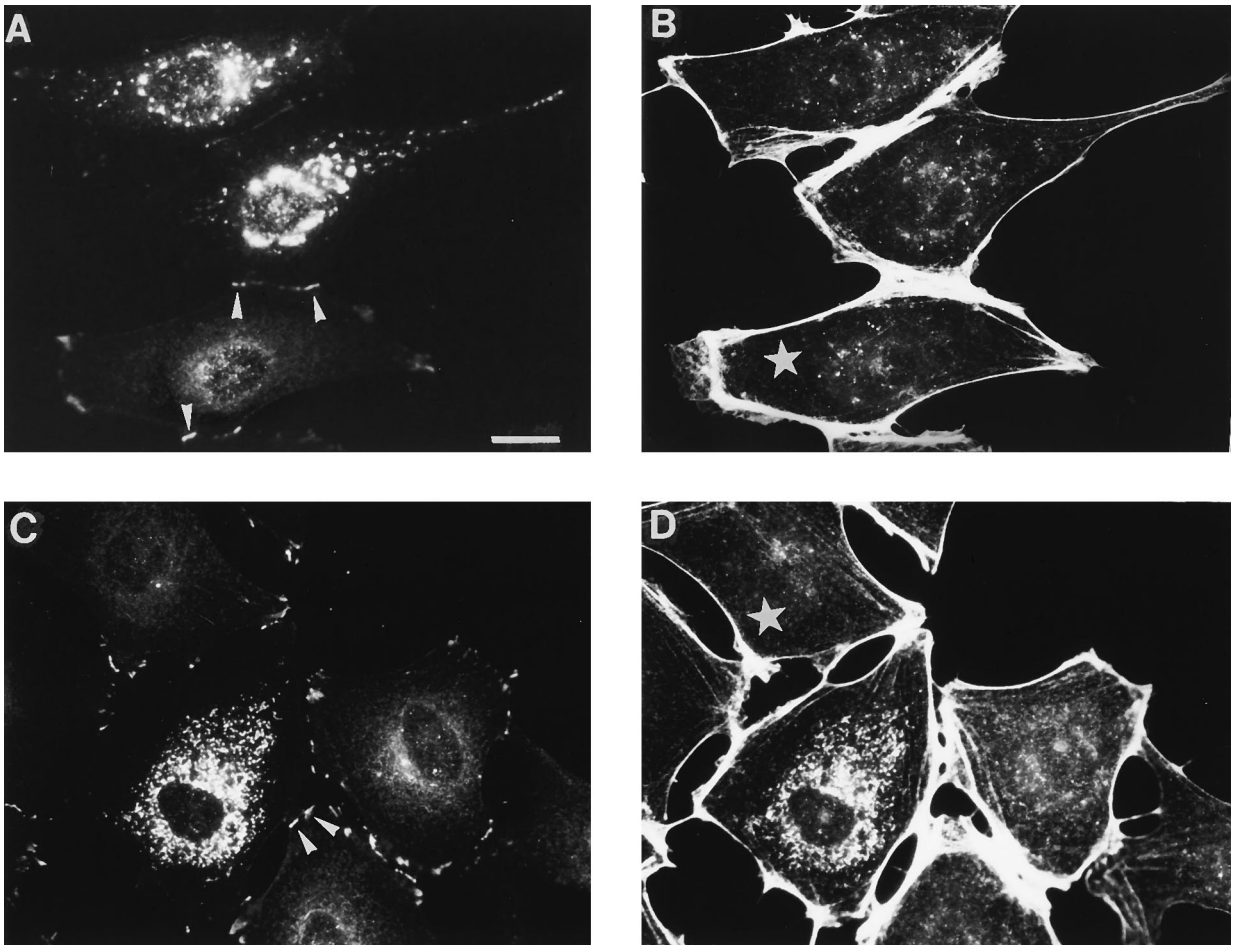

FIG. 5. Distribution of actin and VASP in PtK_2 cells transiently transfected with N-terminally truncated derivatives of *iactA*. Twenty-four hours after the start of transfection, cells were fixed with methanol and processed for double-immunofluorescence microscopy using monoclonal actin antibodies (B and D) and polyclonal VASP antibodies (A and C) followed by incubation with secondary rhodamine- and fluorescein-labelled antibodies. (A and B) Double-immunofluorescence images of cells transfected with plasmid pIA2; (C and D) double-immunofluorescence images of cells transfected with plasmid pIA3. VASP localization in focal contacts of nontransfected cells (arrowheads in panels A and C) is indicated. Note that VASP is depleted from focal contacts in transfected cells. Nontransfected cells in panels B and D (stars) are also shown. Bar, 10 μm.

a to c). Nucleotide sequencing of the amplified fragments confirmed this assumption, indicating that the single repeats are highly conserved among these three *L. ivanovii* strains (data not shown). In a VASP overlay experiment using SDS cell wall extracts, the iActA polypeptides of all three *L. ivanovii* strains bound VASP (Fig. 2C). Interestingly, the different iActA polypeptides seemed to bind VASP more weakly than ActA.

Complementation of an *actA* deletion mutant of *L. monocytogenes* with *iactA*. Transformation of the Δ*actA2* mutant of *L. monocytogenes* with a plasmid carrying the *L. ivanovii iactA* gene (pBPLIVAN) was performed as described in Materials and Methods. The *L. ivanovii* iActA polypeptide was easily detectable by SDS-PAGE as a 190-kDa polypeptide in cell wall extracts derived from the complemented *L. monocytogenes actA* deletion mutant (Fig. 2A, lane c). This indicated that the promoter region of *iactA* is sufficiently induced by PrfA of *L. monocytogenes*, thus replacing its *L. ivanovii* counterpart. Even though affinity-purified antibodies against *L. monocytogenes* ActA were less reactive, these antibodies identified the iActA polypeptide having the same molecular mass of approximately 190 kDa as that detected in the parental strain (compare lanes c and d in Fig. 2). N-terminal microsequencing of the 190-kDa polypeptides derived from both the wild-type ATCC 19119 strain and the complemented *L. monocytogenes* Δ*actA2* mutant gave the sequence SNSTVSTSSNENSNLESD, which corresponds to positions 30 to 47 of the primary sequence. This indicated (i) a signal peptide of 29 aa and (ii) identical processing for the two polypeptides, in agreement with the predicted cleavage site for the signal peptide (6, 12). Upon complementation of the *L. monocytogenes* Δ*actA2* deletion mutant with the *iactA* gene, intracellular actin-based motility could partially be restored in infected tissue culture cells (data not shown). Similar data have previously been published by Gouin et al. (6).

Mitochondrially targeted iActA recruits both F-actin and VASP. The mitochondrial targeting assay was used to assess the ability of iActA to recruit host microfilament proteins to these subcellular organelles (20, 21). PtK_2 cells were transiently transfected with plasmids harboring the full-length *iactA* gene expressed from the promoter of the myeloma proliferative sarcoma virus. The transfected cells were then processed for immunofluorescence microscopy to analyze the distribution of actin, α-actinin, and VASP as described in Materials and Methods. As shown in Fig. 4 by double-immunofluorescence microscopy, the mitochondrially targeted

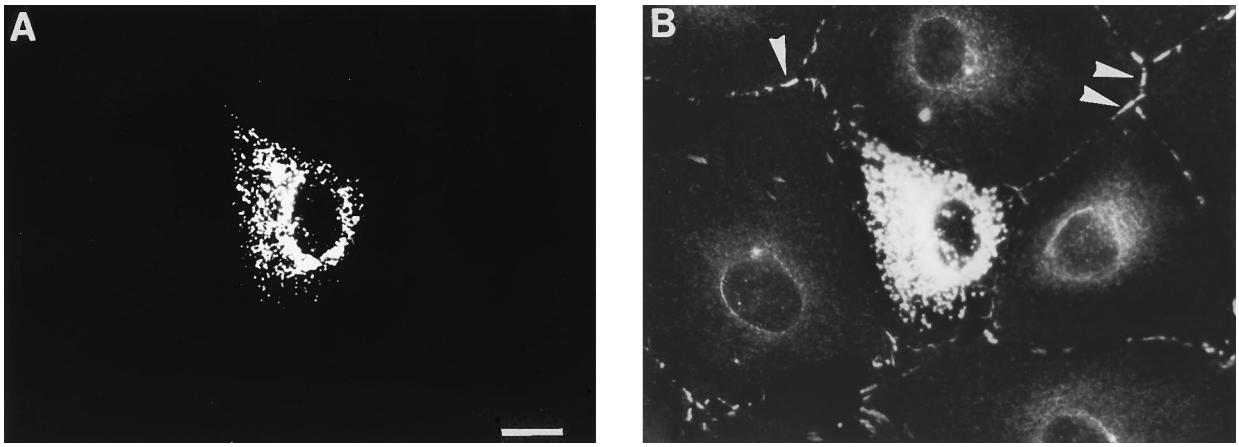

FIG. 6. Colocalization of VASP with mitochondrially targeted hybrid ActA derivatives in PtK_2 cells transiently transfected with plasmid pIA7. At 24 h after the start of transfection, transfected cells were processed for double-immunofluorescence microscopy using monoclonal ActA antibodies (A) and polyclonal VASP antibodies (B). VASP localization in focal contacts of nontransfected cells (arrowheads in panel B) is indicated. Note that methanol fixation reduces diffuse VASP staining in comparison with that in Fig. 4D. Bar, 10 μm.

iActA recruited VASP (Fig. 4D), actin (Fig. 4F), and α-actinin (Fig. 4H).

By analysis of N-terminally truncated ActA constructs in transfected cells, an amino-terminally located, highly positively charged region within the ActA polypeptide required for actin filament recruitment was identified previously (21). A similar positively charged region was identified in iActA between aa 308 and 314 (Fig. 1C). When PtK_2 cells were transfected with an iActA construct starting with amino acid residue 325 (pIA2 in Fig. 1B), no actin was detectable on iActA-decorated mitochondria despite strong staining for VASP (Fig. 5A and B). However, the iActA derivative starting with amino acid residue 290 (pIA3) showed strong staining for both actin and VASP (Fig. 5C and D). Hence, as previously described for *L. monocytogenes* ActA, a sequence of 35 aa within the N-terminal region of iActA, harboring a highly positively charged cluster, is also required for actin filament recruitment.

The proline-rich repeats of *L. ivanovii* iActA bind VASP. In a previous study, it was shown that an *L. monocytogenes* ActA derivative lacking the proline-rich repeats did not recruit VASP, indicating that this repeat region is responsible for VASP binding (21). In order to examine whether the proline-rich repeats of *L. ivanovii* iActA are also involved in VASP binding, we constructed a chimeric ActA derivative by replacing the proline-rich repeats of *L. monocytogenes* ActA with those of *L. ivanovii* iActA (pIA7 in Fig. 1B). When PtK_2 cells were transiently transfected with this chimeric *actA* derivative and analyzed by double-immunofluorescence microscopy, the ActA-decorated mitochondria recruited VASP. Thus, the proline-rich repeat regions of the two ActA polypeptides exhibit similar functions, i.e., the ability to recruit VASP (Fig. 6).

DISCUSSION

In this study, we demonstrated that in the ActA polypeptides of the two pathogenic *Listeria* species, *L. monocytogenes* and *L. ivanovii*, the regions crucial for their activity, i.e., for the recruitment of host microfilament proteins, are preserved and show striking similarities, although the polypeptides have different sizes and display low levels of overall sequence homology. The differences in size (639 versus 1,115 aa) can be attributed largely to the increased number and length of the repeats and a possible duplication of 150 aa within the N terminus of iActA. To determine the regions of iActA that are involved in recruiting host filament proteins, we have used a previously developed mitochondrial targeting assay (20) that directs the iActA polypeptide to mitochondria in transiently transfected PtK_2 cells. In this assay, iActA behaves identically to ActA. Like ActA, iActA spontaneously inserts into mitochondrial membranes and recruits host microfilament proteins to these subcellular organelles, demonstrating that the membrane anchor of iActA can also act as a mitochondrial targeting signal. As previously shown for the ActA polypeptide (21), this property enabled us to dissect the iActA polypeptide and to identify two functional domains within the molecule which are required for recruiting host microfilament proteins. As in ActA, an N-terminally located and highly positively charged cluster is responsible for actin filament recruitment and the central proline-rich repeat region is required for VASP binding. For ActA, cooperative interactions between the proline-rich repeats and the N-terminal region were demonstrated (21). As VASP also binds profilin (23), it was suggested that VASP recruits profilactin to the bacterial surface in proximity to the actin filament nucleation site on the same molecule, thereby promoting efficient actin filament formation (21). Consistent with this model, profilin can accelerate movement of *L. monocytogenes* in *Xenopus* egg extracts (16).

Unlike in ActA, the proline-rich repeats of iActA are absolutely identical in length and to a great extent also in sequence. Among the four proline-rich repeats of ActA, only a sequence consisting of 11 aa is conserved, whereas in iActA of strain ATCC 19119 all eight repeats are highly conserved along their entire sequence of 47 aa. A core sequence of 11 aa is common to all proline-rich repeats of both ActA polypeptides. Since VASP binds to both ActA and iActA, these residues most probably constitute the core binding sequence of VASP. This consensus sequence consists of a negatively charged domain and the proline-rich stretch preceded by an aromatic and an acidic residue [(D/E)FP(P/S)(P/I)PT(D/E)(E/D)EL] (Fig. 1E). Even though substitutions of the amino acid residues serine and isoleucine are apparently tolerated within the polyproline motif, our overlay assay suggests that iActA binds VASP more weakly than ActA. As a potential balance, the iActA polypeptides of different *L. ivanovii* strains harbor up to eight proline-rich repeats compared with four repeats in ActA. Further support for the supposition that the polyproline sequence in-

deed binds VASP comes from microinjection studies which showed that a synthetic peptide containing the sequence DFP PPPTDEELRL interferes with VASP localization to focal adhesion plaques in noninfected cells (21). Indirect evidence that the proline-rich repeats, and as a consequence VASP binding to ActA, are required for efficient bacterial movement stems from microinjection studies showing that the synthetic peptide CFEFPPPPTDE of ActA blocked *Listeria* motility in infected tissue culture cells (29). In support of these observations, Smith and Portnoy (26) showed that in-frame deletions of the proline-rich repeats within the ActA polypeptide markedly impaired *Listeria* motility in infected cells. In a similar experimental approach, these findings were recently confirmed by Lasa et al. (13), who showed that the proline-rich repeat domain of ActA accelerates bacterial movement.

In conclusion, we have demonstrated that the ActA polypeptides of *L. monocytogenes* and *L. ivanovii* harbor at least two highly conserved functional domains involved in actin filament recruitment, leading to efficient intra- and intercellular bacterial movement. Having identified VASP as a cellular factor that directly binds to the proline-rich domains, our future experiments are now directed at identifying the host protein(s) which interact(s) with the N-terminal sequences that we have identified in both ActA and iActA as being essential for actin filament recruitment.

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